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**MicroRNA-27a regulates beta cardiac myosin heavy chain gene
expression by targeting thyroid hormone receptor β 1 in neonatal rat
ventricular myocytes**

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Abstract

MicroRNAs (miRNAs), small non-coding RNAs, are negative regulators of gene expression and play important roles in gene regulation in the heart. To examine the role of miRNAs in the expression of the two isoforms of cardiac myosin heavy chain (MHC) gene, α - and β -MHC, which regulate cardiac contractility, endogenous miRNAs were down-regulated in neonatal rat ventricular myocytes (NRVMs) using lentivirus-mediated siRNA against Dicer, an essential enzyme for miRNA biosynthesis, and MHC expression levels were examined. As a result, Dicer siRNA could down-regulate endogenous miRNAs simultaneously, including miR-16 and -133b and the β -MHC gene but not α -MHC, which implied that specific miRNAs could up-regulate the β -MHC gene. After screening for β -MHC mRNA expression in NRVMs over-expressing of 21 randomly selected miRNAs, miR-27a was found to up-regulate the β -MHC gene but not α -MHC. Moreover, β -MHC protein was down-regulated by silencing of endogenous miR-27a. Through a bioinformatics screening using TargetScanTM, we identified thyroid hormone receptor β 1 (TR β 1), which negatively regulates β -MHC transcription, as a target of miR-27a. Moreover, miR-27a was demonstrated to modulate β -MHC gene regulation via thyroid hormone signaling. These findings suggested that miR-27a regulates β -MHC gene expression by targeting TR β 1 in NRVMs.

1 Introduction

2 MicroRNAs (miRNAs) are negative regulators of gene expression that
3 inhibit the translation or promote the degradation of target mRNAs (36). Mature miRNAs
4 (10 to 24 nucleotides long) are the result of sequential processing of primary transcripts
5 (primary miRNAs) mediated by two RNase III enzymes, Drosha and Dicer (6). Recently, it
6 has been reported that cardiac-specific Dicer-deficient mice presented with cardiac
7 dysfunction, such as cardiac sudden death, dilated cardiomyopathy and heart failure (4, 7),
8 suggesting an essential role of the miRNA-processing machinery in the maintenance of
9 cardiac function. A series of microarray analyses in rodent and human hearts have revealed
10 the profile of miRNA expression in various pathologic conditions, such as cardiac
11 hypertrophy, heart failure, and myocardial infarction, which indicated the involvement of
12 miRNAs in cardiac pathophysiology (5, 27, 29-31, 34, 36). Moreover, genetically modified
13 mice have revealed the effect of specific miRNAs in the heart. For example, mice lacking
14 miR-1-2 suffer from cardiac arrhythmia and congenital malformation, and transgenic mice
15 that over-express miR-195 in the heart or mice lacking miR-133a, a muscle-specific
16 miRNA, present with cardiac dilatation and heart failure (20, 34, 39). These previous
17 studies have revealed the novel role of miRNAs in cardiac development and
18 pathophysiology.

19 Cardiac contractility depends on the expression of two cardiac myosin
20 heavy chain (MHC) genes, α - and β -MHC, which are regulated in an antithetical manner
21 by developmental, physiological, and pathological signals (37). Moreover, the β -MHC gene
22 is up-regulated in response to stress signals causing cardiac hypertrophy and heart failure.

Therefore, a search for factors, including miRNAs, that can regulate β -MHC gene expression may give new findings for heart disease and therapy.

Recently, two groups have revealed that cardiac specific miR-208a, encoded by an intron of the α -MHC gene, is important for the regulation of β -MHC gene expression using miR-208a-deficient mice and transgenic mice that over-express miR-208a under control of the α -MHC promoter (1, 35). Thus, genetically modified mice are a powerful tool for elucidating the final outcome derived from specific miRNAs. However, in vitro screening analyses are still needed to detect the direct effects of individual miRNAs because neurohormonal and hemodynamic effects are considered to strongly influence the gene regulation of β -MHC among others (24, 26), and possibly blur many direct effects of miRNAs in vivo.

Thyroid hormone has a fundamental role in cardiovascular homeostasis in both physiological and pathological conditions, influencing cardiac contractility, heart rate, diastolic function, and systemic vascular resistance through genomic and non-genomic effects (11). These multiple effects are largely mediated by the action of nuclear-based thyroid hormone receptors (8). In particular, α -MHC is increased by tri-iodothyronine (T3), an active form of thyroid hormone, and β -MHC is decreased (8). Moreover, the alterations in thyroid hormone signaling are associated with cardiac pathophysiology, such as hypertrophy and heart failure (8, 11).

In the present study, Dicer siRNA and 21 randomly selected miRNAs were individually transduced into neonatal rat ventricular myocytes (NRVMs) using a lentiviral vector, and MHC gene expression was evaluated. We showed that (1)

down-regulation of Dicer, an essential enzyme for miRNA biosynthesis, globally reduced endogenous miRNAs, which resulted in the down-regulation of the β -MHC gene, and (2) miR-27a was a novel factor that could regulate β -MHC gene expression via thyroid hormone receptor β 1 (TR β 1) in NRVMs.

Materials and Methods

Cell Culture

NRVMs were isolated from 1-day-old Sprague-Dawley rats as described previously (14). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and plated in MULTIWELLTM PRIMARIATM 6-well plates (BECTON DICKINSON) at 37°C in a 5% CO₂ incubator. DNA transduction was carried out 48 h after plating the cells. Cultures were treated with tri-iodothyronine (T5516, Sigma).

Plasmids

MicroRNA (miRNA)-expressing vectors, including miR-1, -15b, -16, -21, -22, -23a, -24, -27a, -29a, -30c, -125b, -126, -130a, -133b, -143, -146, -150a, -195, -199a, -208a, and -424, were constructed using a BLOCK-iTTM Pol II miR RNAi Expression Vector kit (Invitrogen) following the manufacturer's instructions. A control miRNA expressing vector (Cont.miR) was obtained from the kit. For the construction of anti-miR-27a, double-stranded oligonucleotides containing three or six sequences that were

1 completely complementary to miR-27a were inserted into a pMIR-REPORTTM vector
2 (Ambion) at the Pme I site (miR-27a decoy) in accordance with previous studies (3, 9, 23).
3 SiRNA vectors were constructed from pSINsi-mU6 DNATM (Takara Bio.). Double-stranded
4 oligonucleotides were inserted into pSINsi-mU6 DNATM at the BamHI/ClaI sites.
5 Oligonucleotides targeting specific genes and the control (Control siRNA) were as follows:
6 Dicer siRNA 5' -GGAATGGACTCTGAGCTTA- 3'; TRβ1 siRNA1 5' -GGAATGTC-
7 GCTTTAAGAAA- 3'; TRβ1 siRNA2 5' -GGAAGCTGAAGAGAA- 3'; RXRα siRNA1
8 5' -CAAGAGGACAGTACGCAA- 3'; RXRα siRNA2 5' -CCAAGACTGAGACATAC-
9 GT- 3'; Control siRNA 5' -AATAATAATGGGGGGATCC- 3'. All of these constructs
10 were inserted into a pLenti6/V5-D-TOPOTM vector (Invitrogen). The rat TRβ1 gene was
11 amplified and cloned into a pLenti6/V5-D-TOPO vector using the following primers:
12 forward 5' -ATGACAGAAAATGGCCTTCCAGCCT- 3', reverse 5' -TCAGTCCTCA-
13 AAGACTTCCAAGAA- 3'. The following primers were used to amplify and clone a part
14 of the 3'UTR of the rat TRβ1 and THRAP1 and the human TRβ1 and THRAP2 into a
15 pMIR-REPORTTM luciferase vector at the SpeI/HindIII sites in accordance with the
16 manufacturer's instructions: rat TRβ1 forward 5' -GGACTAGTCAGACCATGCATAG-
17 GAAACACCAT-3', reverse 5' -CCCAAGCTTCACCCACATGCATTCCGTTTCCGAA-
18 3'; THRAP2 forward 5' -CCGGAAGCGCTTGCCCTCTGCCT-3', reverse 5' -AGCCC-
19 CAGTGCTAGAT CCTGTACT- 3'; human TRβ1 forward 5' -ACAAGCCCTGGCCC-
20 CTCCTCGACA- 3', reverse 5' - GCACAGTAAAATTCTGTGATAAG- 3'; THRAP1
21 forward 5' -GACTTACTAATGTACTGTACAGA- 3', reverse 5' -ATACAGTAATCT-
22 GTGCCATACTGA- 3'. The rat β-MHC luciferase promoter construct consisted of the

1 firefly luciferase cDNA driven by a 333-bp rat β -MHC promoter sequence (13).

3 **Lentivirus Production and DNA Transduction**

4 Lentiviral stocks were produced in 293FT cells following the
5 manufacturer's protocol (Invitrogen). In brief, virus-containing medium was collected 48 h
6 post-transfection and filtered through a 0.45- μ m filter. One round of lentiviral infection was
7 performed by replacing the medium with virus-containing medium with 8 μ g/ml of
8 polybrene, followed by centrifugation at 2,500 rpm for 30 min at 32°C.

10 **RNA Extraction and Quantitative Real-time PCR (qRT-PCR)**

11 Total RNA was isolated and purified from NRVMs using TRIzol™
12 reagent (Invitrogen), and cDNA was synthesized from 5 μ g of total RNA using
13 SuperScriptII™ reverse transcriptase (Invitrogen) in accordance with the manufacturer's
14 instructions. For qRT-PCR, specific genes were amplified by 40 cycles using SYBR™
15 Green PCR Master Mix (Applied Biosystems). Expression was normalized to the
16 housekeeping gene GAPDH. The primers used were as follows: GAPDH forward 5'
17 -TTGCCATCAACGACCCCTTC- 3', reverse 5' -TTGTCATGGATGACCTTGGC- 3';
18 Dicer forward 5' -ATGCGATTTTGGACTACCTCATAAC- 3', reverse 5' -TCAGCTG-
19 TTAGGAACCTGAGGCTGG- 3'; α -MHC forward 5' -GACACCAGCGCCACCTG- 3',
20 reverse 5' -ATAGCAACAGCGAGGCTCTTTCTG- 3'; β -MHC forward 5' -GGAGCT-
21 CACCTACCAGACAGA- 3', reverse 5' -CTCAGGGCTTCACAGGCATCC- 3'; total
22 MHC forward 5' -GAGGCGGTGCAGGAGTG TAG-3', reverse 5' -ACCTGGGACT-

CGGCAATGTC- 3'; TRβ1 forward 5' -AGCCAGCCACAGCACAGTGA- 3', reverse 5'
-CGCCAGAAGACTGAAGCTTGC- 3'; DDR2 forward 5' -AGTCAGTGGTCAGAGT-
CCACAGC- 3', reverse 5' -CAGGGCACCAGGCTCCATC- 3'. We used TaqMan
MicroRNA AssaysTM (Applied Biosystems) to determine the expression levels of miRNAs
in accordance with the manufacturer's instructions.

Northern Blotting Analysis

Northern blotting analysis was performed as described previously (25). In
brief, small RNA fractions were isolated from total RNA using a *mirVana* miRNA
IsolationTM kit (Ambion). The small RNA fractions, 5 µg, were separated by
electrophoresis using a 15% polyacrylamide (19:1) denaturing gel and transferred to nylon
hybridization membrane (Hybond-NX; Amersham) using a semidry electroblotter (Bio-Rad,
Hercules, CA, USA) at 20 V for 30 min at 4°C. Cross-linking of RNA was performed using
0.16 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma) in 0.13 M
1-methylimidazole (Sigma) at pH 8.0 for 2 h at 60°C. Templates to make probes for
miR-16, miR-133b, and U6 were prepared using a *mirVana* miRNA Probe Construction
KitTM (Ambion) with the following oligonucleotides: miR-16 5' -TAGCAGCAC-
GTAAATATTGGCGCCTGTCTC- 3'; miR-133b 5' -TTGGTCCCCTTCAACCAGAC-
CTGTCTC- 3'; U6 5' -CGATACAGAGAAGATTAGCATGGCCCCTGCCCTGTCTC- 3'.

Luciferase Assay

For luciferase reporter assays, constructs were transiently transfected using Fugene 6TM (Roche) into 293FT cells or using Lipofectamine 2000TM (Invitrogen) into NRVMs at the following concentrations: 0.1 µg of firefly luciferase reporter gene, 0.01 µg of pRL-TKTM Renilla reniformis luciferase control plasmid (Promega), and 0.1 µg of BLOCK-iT Pol II miR RNAi Expression VectorTM encoding the appropriate miRNA or the control. At 24 h after transfection, both luciferase activities were measured using a dual luciferase reporter assay system (Toyo Ink Co.). Firefly luciferase activity was normalized for transfection efficiency by measuring that of renilla reniformis control activity in accordance with the manufacturer's instructions.

Western Immunoblotting Analysis

Immunoblotting analysis was performed using standard procedures as described previously (38). Cultured cells were homogenized in lysis buffer consisting of 100 mM Tris-HCl, pH 7.4, 75 mM NaCl and 1% TritonTM X -100 (Nacalai Tesque). The buffer was supplemented with Complete MiniTM protease inhibitor (Roche), 0.5 mM NaF, and 10 µM Na₃VO₄ just prior to use. The protein concentration was determined using a BCA protein assay kit (BIORAD). A total of 2 or 10 µg of protein was fractionated using NUPAGETM 4-12% Bis-Tris (Invitrogen) gels and transferred to PROTRANTM nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1×PBS containing 5% non-fat milk for 1 h and incubated with the primary antibody overnight at 4°C. Following a wash step in 0.05% T-PBS (1×PBS and 0.05% Tween-20), the membrane was incubated with the secondary antibody for 1 h at 4°C. After washing the membrane in 0.05% T-PBS,

the immunocomplexes were detected using ECL[™] Western Blotting Detection Reagent (Amersham Biosciences). The primary antibodies used were: anti-GAPDH (Cell Signaling Technology), 1:1,000; anti-Dicer (sc-25117, Santa Cruz Biotechnology, Inc.), 1:500; anti- α -MHC (ab50967, Abcam), 1:20,000; anti-skeletal slow myosin (M8421, Sigma) for detecting β -MHC, 1:20,000; anti-TR β 1 (sc-738, Santa Cruz Biotechnology, Inc.), 1:500; and anti-RXR α (sc-553, Santa Cruz Biotechnology, Inc.), 1:500. As secondary antibodies, anti-rabbit, anti-mouse, and anti-goat IgG (GE Healthcare) as used at a dilution of 1:2,000. Immunoblots were detected using an LAS-1000 system (FUJI FILM).

Statistics

Data are presented as means \pm S.E. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests where appropriate, with a probability value of <0.05 was taken to indicate significance.

Results

Dicer siRNA can down-regulate endogenous miRNAs and the β -MHC gene in NRVMs

To investigate the effects of miRNAs on cardiac MHC gene expression, we first tried to suppress the function of endogenous miRNAs in NRVMs. Because Dicer is an essential enzyme for miRNA biosynthesis, it was hypothesized that the down-regulation of Dicer could result in a global reduction of miRNAs, which equates to a loss-of-function of miRNAs. Dicer siRNA was transduced into NRVMs using a lentiviral vector, which resulted in the down-regulation of both Dicer mRNA and protein in serum-containing

conditions (Figs. 1A and B). Moreover, Dicer siRNA could down-regulate ubiquitously expressed miR-16 and muscle-specific miR-133b at the same time (Fig. 1C), suggesting the global reduction of endogenous miRNAs in NRVMs. The MHC gene has two isoforms, α - and β -MHC, and the gene expression of both isoforms was assessed in NRVMs into which Dicer siRNA was transduced. As a result, Dicer siRNA decreased both mRNA and protein levels of β -MHC but not those of α -MHC, while total MHC mRNA levels were not altered (Fig. 1D and E). These results indicated that a loss-of-function of miRNAs could suppress β -MHC gene expression specifically in NRVMs in serum-containing conditions, and there were specific miRNAs that can up-regulate β -MHC gene expression.

miR-27a can up-regulate the β -MHC gene in NRVMs

To identify miRNAs that can up-regulate β -MHC gene expression, 21 randomly selected miRNAs were individually transduced into NRVMs using a lentiviral vector, and mRNA levels of β -MHC were examined in serum-containing conditions. As a result, miR-27a was found to increase β -MHC mRNA levels most strongly among the miRNAs assessed, while several miRNAs, such as miR-22, -23a, 29a, and -30c, could increase β -MHC levels to a lesser extent (Fig. 2A). The lentiviral vector could express miR-27a abundantly in NRVMs (Fig. 2B). Next, to study whether endogenous miR-27a was expressed in cardiac myocytes, miR-27a levels were examined both in NRVMs and cardiac fibroblasts. The appropriate separation between both types of cells was confirmed by detecting the mRNA of β -MHC, a myocyte-specific marker, or collagen receptor

Discoidin Domain Receptor 2 (DDR2), a cardiac fibroblast-specific marker (2) (Fig. 2C). As a result, miR-27a was found to be expressed in cardiac myocytes but not cardiac fibroblasts (Fig. 2D). Further examination of miR-27a confirmed that over-expression of miR-27a in NRVMs could increase β -MHC gene expression both at mRNA and protein levels in serum-containing conditions but did not affect those of α -MHC (Figs. 2E and F). These findings suggested that the up-regulation of miR-27a could up-regulate β -MHC gene expression in cardiac myocytes.

Loss-of-Function of miR-27a Decreases β -MHC Protein Levels in NRVMs

To examine the direct effect of miR-27a on β -MHC gene regulation, β -MHC protein expression was also assessed in NRVMs in which miR-27a function was suppressed. NRVMs infected with a lentiviral vector were used in which a 3'UTR with three or six tandem miR-27a decoy sequences complementary to miR-27a was linked to the luciferase reporter gene in accordance with previous studies (Fig. 3A) (3, 9, 23). The complementary sequences acted as a decoy, sequestering endogenous miR-27a. When miR-27a was transfected into 293FT cells along with miR-27a decoy*6, the luciferase activity of the decoy was significantly reduced (Fig. 3B). On the other hand, miR-1, -21, -133b, and -208a did not affect luciferase activity (Fig. 3B). Next, miR-27a decoys were transfected into NRVMs, which resulted in the reduction of luciferase activity. These results indicated that miR-27a decoys could specifically bind to endogenous miR-27a (Fig. 3C). When miR-27a decoys were transduced into NRVMs using a lentiviral vector, β -MHC protein levels decreased but those of α -MHC did not change in serum-containing

conditions (Fig. 3D and E). These results suggested that miR-27a is directly involved in β -MHC gene regulation in NRVMs.

Target prediction of miR-27a

To find targets of miR-27a that can regulate β -MHC gene expression, a search was made for putative target genes of miR-27a using Target ScanTM, a bioinformatics tool for miRNA target prediction. Because miRNAs suppress gene expression, it was supposed that over-expression of miR-27a could down-regulate target genes that can negatively regulate β -MHC expression, which might result in up-regulation of the β -MHC gene. Transcription factors that can negatively regulate β -MHC gene transcription or related co-regulators were expected to be involved in this up-regulation of the β -MHC gene. There are several transcription factors that bind to the rat β -MHC promoter, and one of negative regulators of β -MHC, thyroid hormone receptor β 1 (TR β 1) (12), was predicted as one of targets of miR-27a according to Target ScanTM (Fig. 4A). TR β 1 binds to a negative thyroid hormone responsive element in the β -MHC promoter and negatively regulates β -MHC transcription (18, 22). To test whether the putative target sequence in the 3'UTR of TR β 1 could mediate translational repression, the 3'UTR of TR β 1 was inserted into a luciferase reporter construct, which was transfected into 293FT cells, and CMV-driven miR-27a consequently decreased the luciferase activity of the construct (Fig. 4A). The human TR β 1 mRNA was predicted to have other binding sites for miR-27a, and the luciferase activity of a human TR β 1 3'UTR reporter was decreased significantly by

miR-27a (Fig. 4B). These findings suggested that the binding sites of miR-27a in the 3'UTR of TR β 1 could mediate translational repression by miR-27a. Thyroid hormone receptors form a homo-dimer, or hetero-dimer with retinoid X receptors and associate with nuclear proteins such as thyroid hormone receptor associated proteins (16). Retinoid X receptor α (RXR α) and thyroid hormone receptor associated protein 1 and 2 were predicted as targets of miR-27a according to Target ScanTM. A recent study revealed that miR-27a can target RXR α in rat hepatic cell lines (17), while thyroid hormone receptor associated protein 1 and 2 could not be demonstrated as targets of miR-27a (Figs. 4C and D).

miR-27a targets thyroid hormone receptor beta 1 (TR β 1)

To test whether miR-27a could actually target TR β 1, TR β 1 gene expression was examined in NRVMs transduced with miR-27a or miR-27a decoys. As a result, miR-27a decreased TR β 1 protein levels without affecting TR β 1 mRNA levels in NRVMs (Figs. 5A and B), and miR-27a decoys increased TR β 1 protein levels (Fig. 5C). These findings indicated that TR β 1 is a target of miR-27a. Next, it was examined whether RXR α siRNA could increase β -MHC gene expression in NRVMs. RXR α siRNAs did not increase β -MHC protein levels in serum-containing conditions (Fig. 5D), whereas TR β 1 siRNAs increased β -MHC protein levels (Fig. 5E). Because miR-27a could up-regulate the β -MHC gene in serum-containing conditions, these results indicated that the up-regulation of β -MHC by miR-27a resulted mainly from the down-regulation of TR β 1 rather than RXR α .

Over-expression of miR-27a attenuates thyroid hormone effect

. To examine whether miR-27a regulates β -MHC gene expression via TR β 1, miR-27a- or miR-27a decoy-transduced NRVMs were treated with tri-iodothyronine (T3), a ligand of thyroid hormone receptors, and β -MHC protein expression was assessed. Treatment of NRVMs with 10 nM T3 could significantly up-regulate α -MHC and down-regulate β -MHC protein levels (Fig. 6A), which was the same result as the previous studies (10, 18, 21). Over-expression of miR-27a attenuated the effect of T3, which can decrease β -MHC protein levels but did not affect those of α -MHC (Fig. 6A). The same result was obtained as that with TR β 1 siRNAs (Fig. 6B). RXR α siRNAs down-regulated β -MHC protein in the absence of T3, whereas in the presence of T3 β -MHC protein levels did not change (Fig. 6C).

miR-27a regulates β -MHC protein expression via TR β 1

To confirm the specific effect of miR-27a on thyroid hormone signaling in β -MHC gene expression, β -MHC protein levels were examined in NRVMs transduced with miR-27a decoys. As a result, miR-27a decoys facilitated the down-regulation of β -MHC protein by 10 nM T3 treatment (Fig. 7A), which was the opposite to that of miR-27a over-expression. Next, to elucidate the effects of up-regulation of TR β 1 on β -MHC gene expression, the rat TR β 1 gene was transduced into NRVMs using a lentiviral vector (Fig. 7B), and β -MHC protein expression was assessed. As a result, over-expression of TR β 1 promoted the down-regulation of β -MHC protein in the presence of 10 nM T3, while in the

absence of T3, β -MHC gene expression did not change significantly (Figs. 7C and D), which was the same result as that with miR-27a decoys (Fig. 7A). On the other hand, TR β 1 over-expression could decrease the luciferase activity of the β -MHC promoter construct, which contained a 333-bp rat β -MHC promoter sequence even in the absence of T3 (Fig. 7E). In serum-containing conditions, TR β 1 also down-regulated β -MHC protein levels but did not change α -MHC protein levels (data not shown), which was the same result as with miR-27a decoys (Figs. 3D and E). These findings indicated that miR-27a regulates β -MHC gene expression via TR β 1.

Discussion

Extensive research into the functions of miRNAs in the cardiovascular system has been performed. However, it is challenging to distinguish the direct effects of a specific miRNA from a range of indirect consequences because a miRNA may down-regulate several hundred target genes at the same time. In *in vivo* studies, many environmental factors, such as cytokines, neurohormones and hemodynamics, or interactions with interstitial components are considered to strongly affect the gene regulation of cardiac myocytes, which makes it more difficult to evaluate the direct effects of individual miRNAs. Therefore, in the present study, it was attempted to examine the direct effects of miRNAs on cardiac gene regulation using primary cultures of NRVMs, in which the environmental factors or the interactions with interstitial components were limited.

We first tried to suppress global miRNA function in NRVMs by siRNA

1 against Dicer, an essential enzyme for miRNA biosynthesis, and elucidate the effects on
2 cardiac MHC gene expression. It had been controversial whether Dicer siRNA could
3 down-regulate Dicer itself because gene suppression by siRNAs requires processing by
4 Dicer. The present data revealed that Dicer siRNA could down-regulate both Dicer and
5 mature miRNAs, including ubiquitous miR-16 and muscle-specific miR-133b, at the same
6 time in NRVMs, suggesting the global reduction of miRNAs by Dicer siRNA.

7 The present study showed that Dicer siRNA could down-regulate the
8 β -MHC gene even though Dicer siRNA did not completely delete mature miRNAs. A
9 previous study has demonstrated that cardiac-specific Dicer-deficient mice, in which
10 mature miRNAs are globally reduced but can be detected, die shortly after birth presenting
11 cardiac dysfunction (4). These findings suggested that miRNAs were relatively stable in
12 this expression system, and the dysregulation of miRNA machinery could significantly
13 affect cellular function and gene regulation in the heart. However, it remained unclear
14 whether the observed events, resulting from Dicer knock-down or knock-out, were derived
15 from the global reduction of miRNAs or the dysregulation of Dicer itself or both.

16 Targeted Dicer deletion through use of a tamoxifen-inducible Cre
17 recombinase in the hearts of 8-week-old mice can result in the up-regulation of β -MHC
18 mRNA levels (7), whereas β -MHC protein levels in cardiac specific Dicer-deficient mice,
19 which die shortly after birth, are unchanged (4). The present data demonstrated that Dicer
20 siRNA could down-regulate the β -MHC gene. These differences in results might be derived
21 from the age at which β -MHC gene expression is different between perinatal and adult
22 mice or the neurohormonal and hemodynamic effect in vivo, which are considered to

1 strongly influence the regulation of genes such as β -MHC (24, 26) but have minimally
2 effect in vitro.

3 We demonstrated that thyroid hormone receptor $\beta 1$ (TR $\beta 1$) is a novel
4 target gene of miR-27a. Among the predicted genes as targets of miR-27a by Target
5 ScanTM, TR $\beta 1$ was clearly one that could be involved in the negative regulation of β -MHC
6 gene expression. TR $\beta 1$ has been already reported as a negative regulator of β -MHC
7 transcription (12, 18). In the present data, over-expression of miR-27a decreased TR $\beta 1$
8 protein levels without affecting mRNA levels. Moreover, miR-27a suppressed the
9 luciferase activity of the TR $\beta 1$ 3'UTR luciferase reporter construct, while miR-27a decoys
10 increased TR $\beta 1$ protein expression. These findings suggested that miR-27a could regulate
11 TR $\beta 1$ post-transcriptionally.

12 Target ScanTM also predicted thyroid hormone receptor associated protein
13 1 and 2 and retinoid X receptor α (RXR α) as the targets of miR-27a. Thyroid hormone
14 receptors form a hetero-dimer with retinoid X receptors and associate with nuclear proteins,
15 such as thyroid hormone receptor associated proteins (16). However, the 3'UTR luciferase
16 reporter assay did not show that miR-27a could target thyroid hormone receptor associated
17 protein 1 or 2. RXR α has been reported as the target of miR-27a (17), but in the presence of
18 serum, RXR α siRNAs did not alter β -MHC protein levels, while TR $\beta 1$ siRNA could
19 increase β -MHC protein levels in a same manner as miR-27a. These results suggested that
20 miR-27a regulates β -MHC gene expression predominantly via TR $\beta 1$ in NRVMs.

21 Here, we demonstrated that miR-27a could modulate thyroid hormone

1 signaling in β -MHC gene expression. The present study demonstrated that the
2 down-regulation of TR β 1 by miR-27a over-expression or TR β 1 siRNA attenuated the
3 effect of 10 nM T3, which could down-regulate β -MHC protein levels. On the other hand,
4 miR-27a decoy enhanced the effect of T3 treatment on β -MHC protein expression in a
5 same manner as TR β 1 over-expression. MHC gene expression is strongly regulated by
6 thyroid hormone and receptors. Thyroid hormone receptors have four main isoforms, α 1,
7 α 2, β 1 and β 2 (19). Isoforms α 1, α 2, and β 1 are expressed differentially but rather
8 ubiquitously, while isoform β 2 exhibits restricted tissue distribution, being found mainly in
9 the pituitary gland (15, 19, 28). TR β 1 regulates β -MHC transcription in NRVMs but not
10 α -MHC (18). The present data also revealed that α -MHC protein levels were not affected
11 by TR β 1 in NRVMs. Moreover, TR α 1 and α 2 cannot be directly involved in the regulation
12 of β -MHC transcription in NRVMs (18). These findings suggested that miR-27a can
13 modulate thyroid hormone signaling specifically in β -MHC gene regulation via TR β 1 in
14 NRVMs. In the absence of T3, over-expression of TR β 1 or miR-27a decoy did not
15 significantly decrease either β -MHC mRNA or protein levels. It has been reported that
16 β -MHC transcription can be suppressed by TR β 1 over-expression even in the absence of
17 T3 in NRVMs (18), and unliganded TR β can suppress basal transcription (32). In these
18 previous studies, β -MHC transcription was evaluated using a CAT promoter assay (18) or
19 by a cell-free transcription assay (32). The present study also showed that even in the
20 absence of T3, TR β 1 over-expression could decrease the luciferase activity of the β -MHC
21 promoter construct, which contained a 333-bp rat β -MHC promoter sequence, but not

1 β -MHC mRNA and protein levels. Therefore, mRNA and protein stability or promoter
2 regulation might be different between reporter genes and the β -MHC gene.

3 In serum-free conditions, or T3-free conditions in this study, the effect of
4 TR β 1 on β -MHC gene regulation was very weak, whereas TR β 1 significantly changed
5 β -MHC expression levels, which suggested that factors in the serum could modulate
6 β -MHC gene regulation by TR β 1. Serum-containing medium used in this study was found
7 to contain T3 at a concentration of 1.12 ± 0.12 nM (data not shown). According to a
8 previous report, 1 and 10 nM T3 can both decrease β -MHC mRNA levels to a minimum in
9 cardiomyocytes (18). These findings suggested that T3 in the serum might modulate
10 β -MHC gene regulation by miR-27a, although there was a possibility that other factors
11 might also be involved in this gene regulation.

12 In summary, miR-27a can regulate β -MHC gene expression by targeting
13 TR β 1 in NRVMs.

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Figure Legends

Figure. 1 *Dicer siRNA down-regulates endogenous miRNAs and β -MHC gene levels.*

Assays were performed 72 h (*A through D*) or 96 h (*E*) after transduction with Dicer siRNA or control siRNA (Cont.siRNA) into NRVMs using a lentiviral vector. *A* and *B*, mRNA (*A*) and protein (*B*) levels of Dicer. *C*, Endogenous miR-16 and miR-133b were detected by northern blotting analysis. *D* and *E*, mRNA and protein levels of α -MHC, β -MHC, and total MHC were detected by qRT-PCR (*D*) and immunoblotting (*E*). Representative data are presented as mean \pm S.E. for three independent experiment (*, $P < 0.05$; ***, $P < 0.001$ versus Cont. siRNA).

Figure. 2 *miR-27a up-regulates β -MHC gene expression.* Assays were performed 72 h

after transduction with individual miRNAs or miR-control (Cont.miR) into NRVMs using a lentiviral vector. NRVMs were cultured in serum-containing medium. *A*, mRNA levels of β -MHC in NRVMs transduced with individual miRNAs were detected by qRT-PCR. *B*, Expression levels of miR-27a or miR-195 in NRVMs transduced with miR-27a or Cont.miR using a lentiviral vector. *C*, mRNA levels of myocyte-specific β -MHC (*white bar*) and cardiac fibroblast-specific DDR2 (*black bar*) both in NRVMs and cardiac fibroblasts. The bar graph indicates values expressed as relative β -MHC (scale; *left Y axis*) and DDR2 (scale; *right Y axis*) mRNA levels compared with that of untreated NRVMs. *D*, Endogenous miR-27a levels in NRVMs and cardiac fibroblasts were detected by qRT-PCR. *E*, mRNA levels of α - and β -MHC were detected by qRT-PCR. Data are presented as mean

± S.E. for three independent experiments (*, P<0.05 versus Cont.miR). *F*, A total of 2 µg of protein was used for immunoblotting of α- and β-MHC. Data are representative of three independent experiments. In *A* and *C*, the bar graph indicates values expressed as relative mRNA levels compared with Cont.miR.

Figure. 3 *miR-27a decoys decrease β-MHC protein levels.* *A*, Structure of “miR-27a decoy”. *B*, 293FT cells were transfected with a luciferase decoy construct (miR-27a decoys or control decoy) along with an expression plasmid for individual miRNAs or miR-control (Cont.miR). *C*, miR-27a decoys and control decoy (Cont.decoy) were transfected into NRVMs. The bar graph indicates values expressed as relative luciferase activity compared with that of the control. Data presented as mean ± S.E. of three independent experiments (*, P<0.05; **, P<0.01; ***, P<0.001 versus control). *D* and *E*, NRVMs were transduced with miR-27a decoys using a lentiviral vector in serum-containing medium. Assays were performed 72 h after transduction. A total of 2 µg of protein was used for immunoblotting of β-MHC (*D*) and α-MHC (*E*). Data are representative of three independent experiments.

Figure. 4 *Target prediction of miR-27a associated with negative TRE in the β-MHC promoter.* The predicted binding sites of miR-27a in the 3'UTR of predicted target gene. 293FT cells were transfected with each of the 3'UTR luciferase construct and an expression plasmid for miR-27a or miR-control (Cont.miR). Rat TRβ1 (*A*), human TRβ1 (*B*), human thyroid hormone receptor associated protein 1 (THRAP1) (*C*), and rat THRAP2 (*D*). Data

are presented as mean \pm S.E. of three independent experiments (*, $P < 0.05$; ***, $P < 0.001$ versus Cont.miR).

Figure. 5 *miR-27a targets TR β 1*. Assays were performed 72 h after transduction into NRVMs in serum-free conditions. TR β 1 mRNA and protein levels were detected by qRT-PCR (A) and immunoblotting (B and C), respectively. A total of 10 μ g of protein was used for immunoblotting. D and E, MHC (D and E), RXR α (D) and TR β 1 (E) protein were detected by immunoblotting in NRVMs transduced with RXR α (D) or TR β 1 siRNAs (E) in serum-containing conditions. A total of 2 μ g of protein was used for immunoblotting. In A, data are presented as mean \pm S.E. of three independent experiments In B through E, data are representative of three independent experiments.

Figure. 6 *Over-expression of miR-27a attenuates thyroid hormone effect*. Treatment with 10 nM T3 was started 24 h after transduction and continued for 48 h. A though C, A total of 2 μ g of protein was used for immunoblotting of MHC in NRVMs transduced with miR-27a (A), TR β 1 siRNAs (B) or RXR α siRNAs (C). Data are representative of three independent experiments.

Figure. 7 *miR-27a regulates β -MHC protein expression via TR β 1*. A though C, Treatment with 10 nM T3 was started 24 h after transduction and continued for 48 h. A total of 2 μ g of protein was used for immunoblotting of MHC (A and C) and 10 μ g of protein for TR β 1 (B).

1 Data are representative of three independent experiments. *D*, The luciferase construct
2 contained the 333-bp sequence upstream of the translation initiation site of rat β -MHC.
3 293FT cells were transfected with the luciferase construct and an expression plasmid for rat
4 TR β 1 or an empty plasmid with or without 10 nM T3. T3 treatment was started 24 h after
5 transfection, followed by assays 24 h after treatment. The bar graph indicates values
6 expressed as relative luciferase activity compared with that for an empty plasmid without
7 T3 treatment. *E*, β -MHC mRNA was detected by qRT-PCR in NRVMs transduced with
8 TR β 1 or empty vector using a lentiviral vector with or without 10 nM T3. T3 treatment was
9 started 24 h after transduction, followed by RNA extraction 72 h after treatment. Data are
10 presented as mean \pm S.E. for three independent experiments.

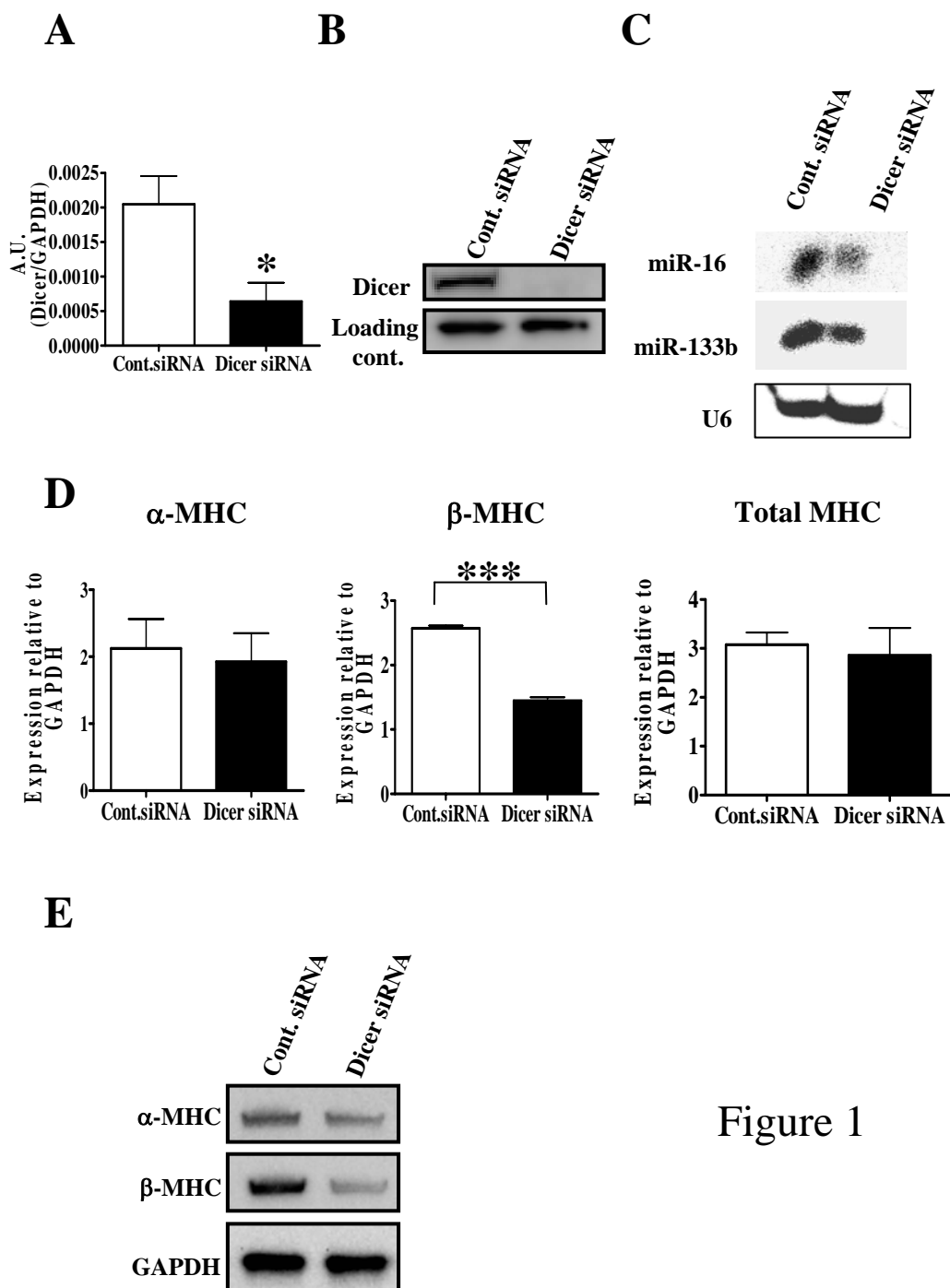


Figure 1

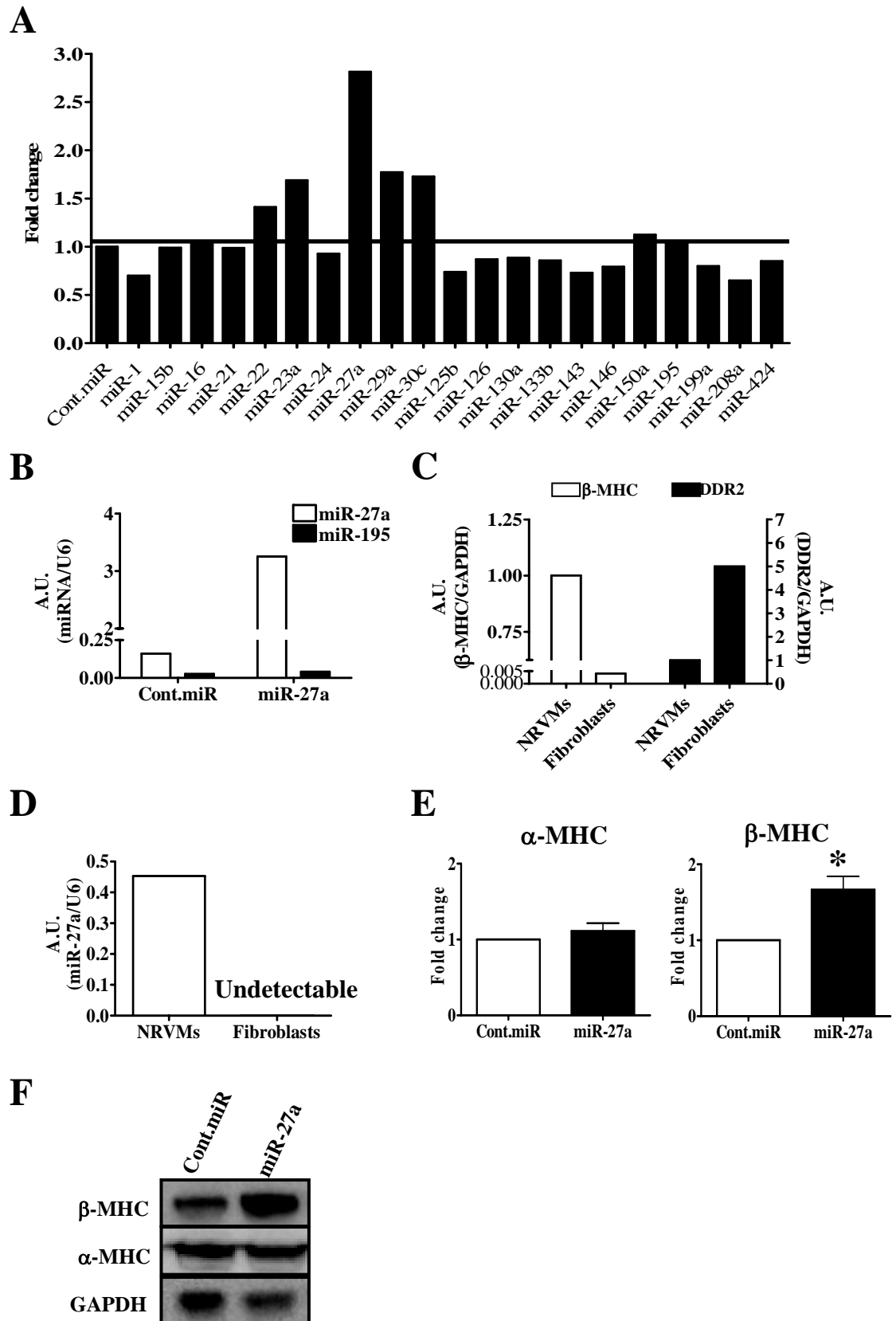


Figure 2

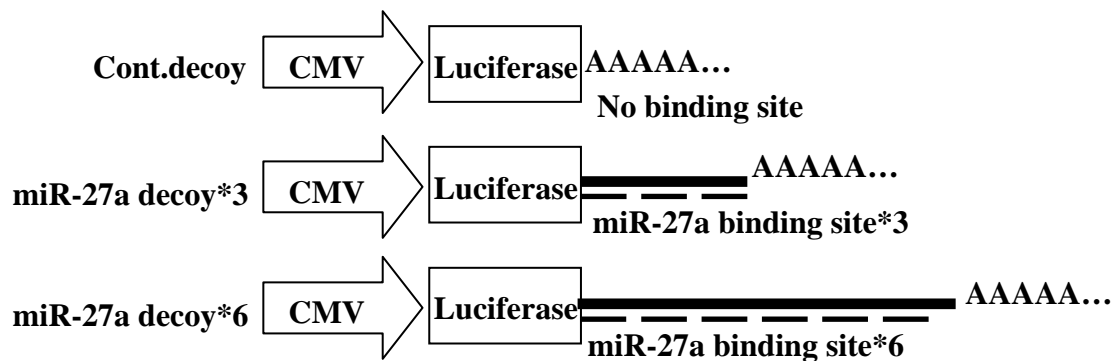
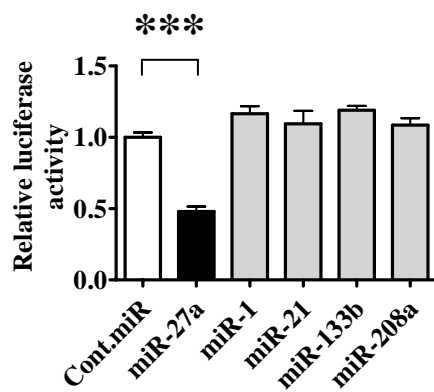
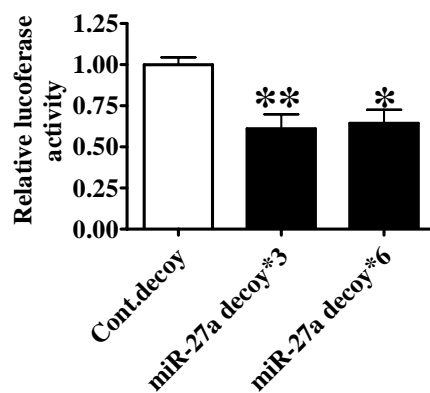
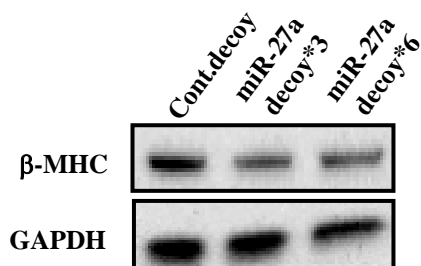
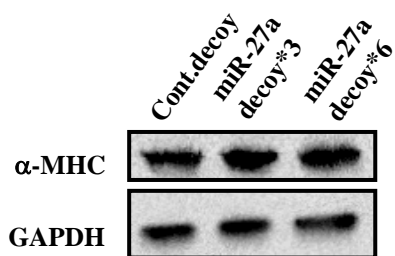
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Figure 3

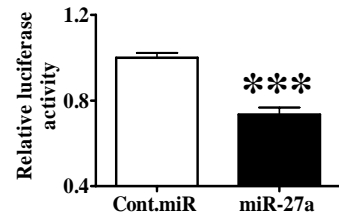
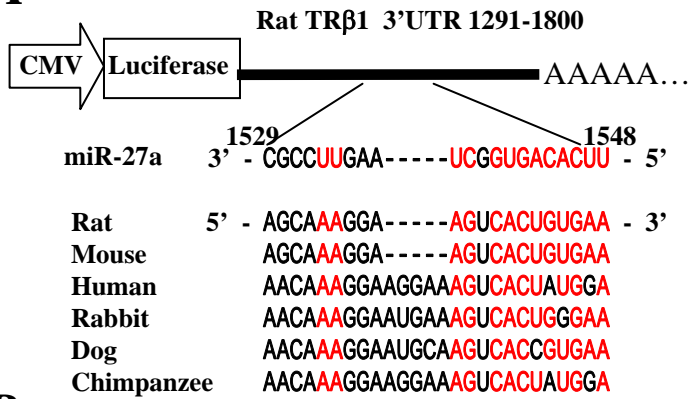
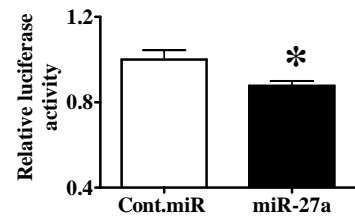
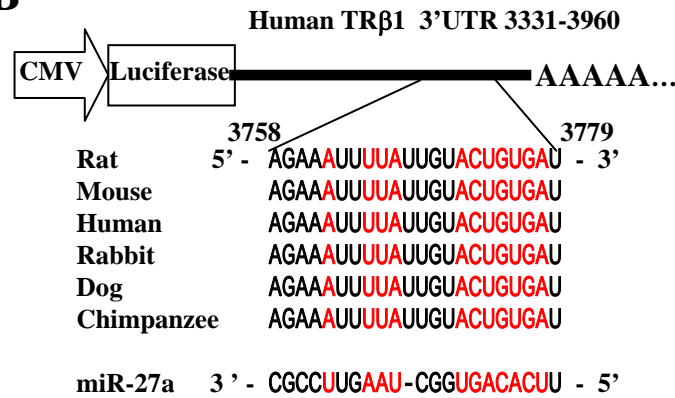
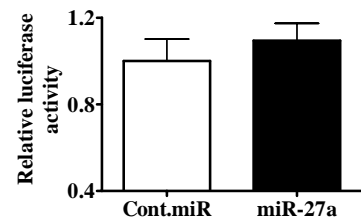
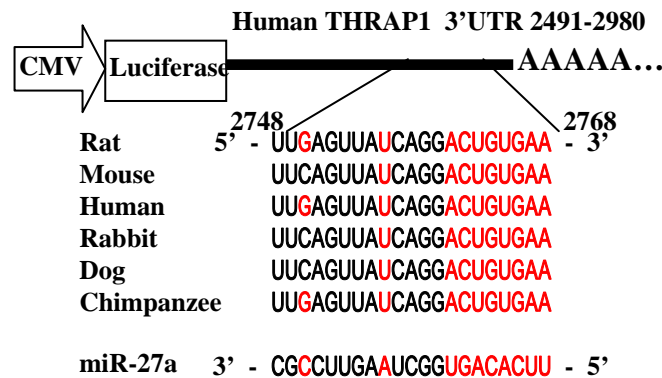
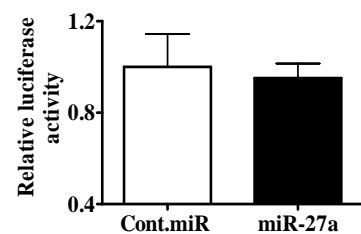
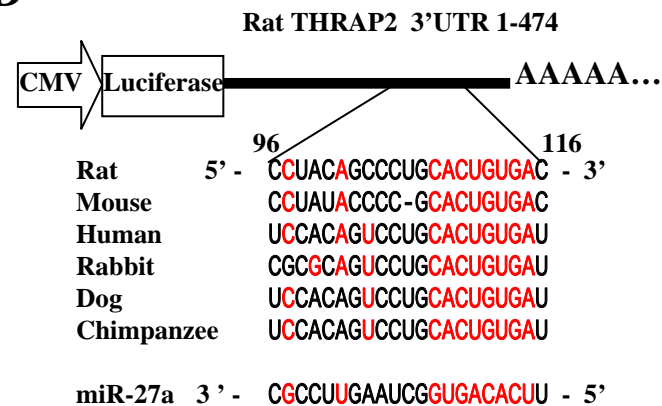
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Figure 4

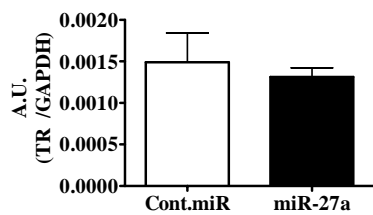
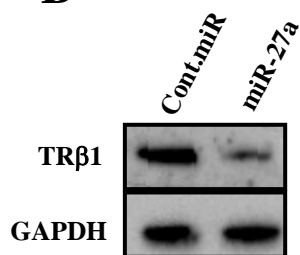
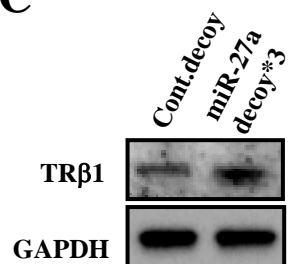
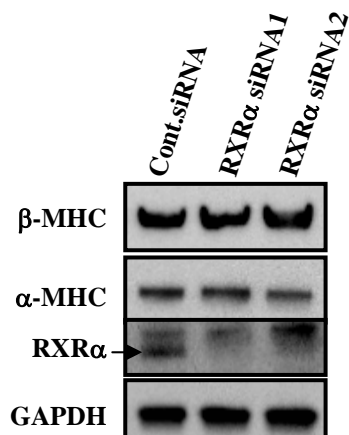
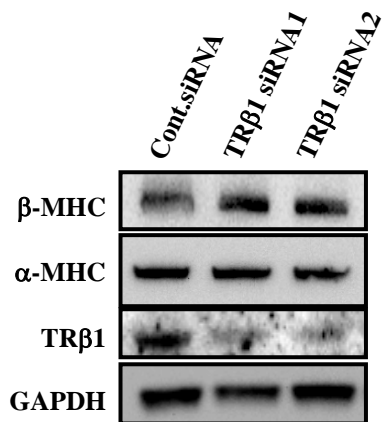
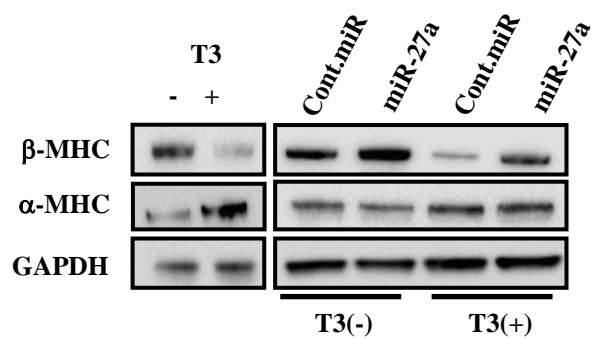
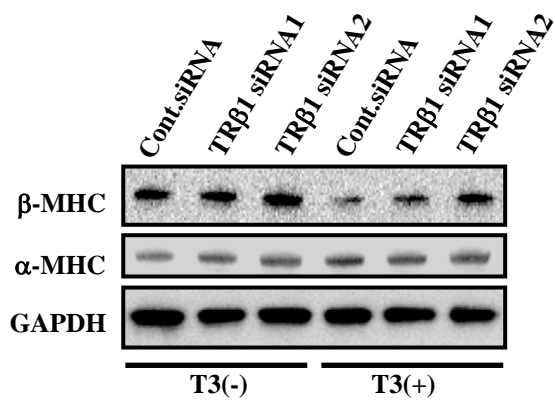
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Figure 5

A



B



C

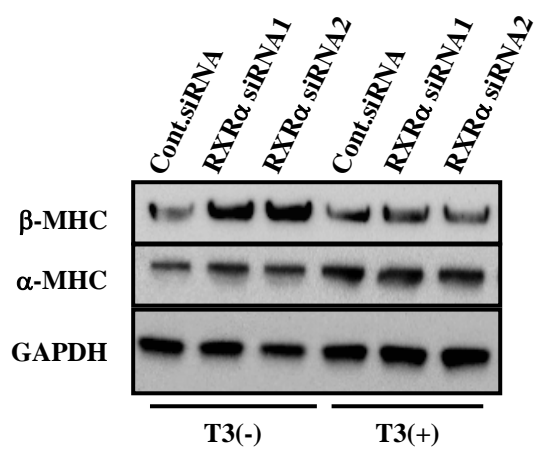


Figure 6

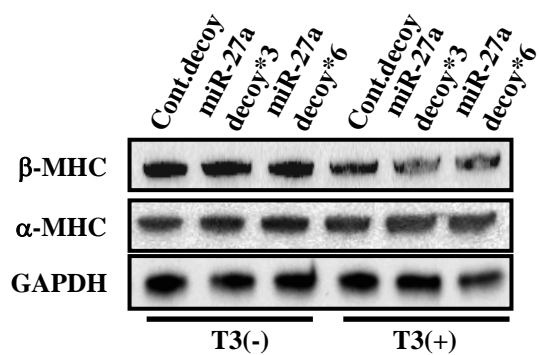
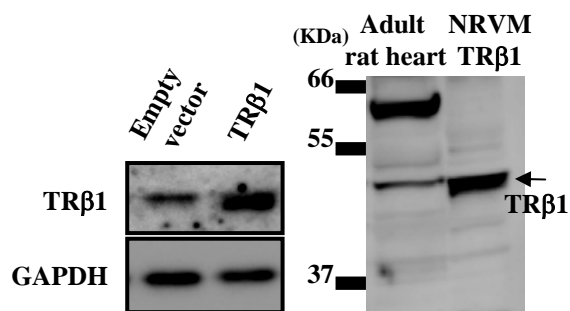
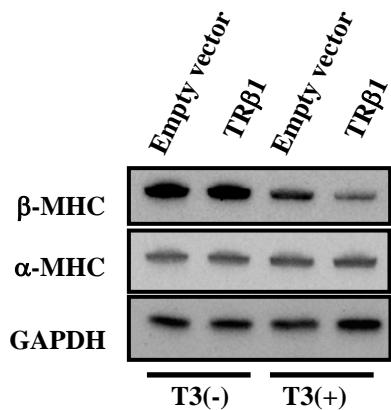
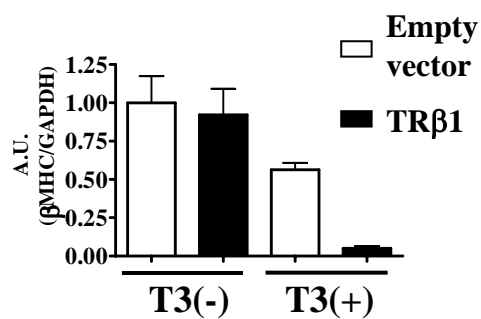
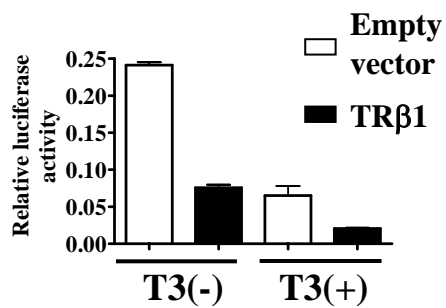
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Figure 7